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# THE ROLE OF $P_i$ IN GLYCOLYTIC INHIBITION OF CALCIUM ION UPTAKE BY ELD ASCITES TUMOR CELLS

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## Summary

1. In contrast to previous investigations at 25°C [1], glucose was shown to support $^{45}Ca^{2+}$ uptake at 37°C in intact ELD ascites tumor cells.

2. Intact ascites tumor cells in vitro accumulated up to 5.0 μmol of $^{45}Ca^{2+}$ per g cells dry wt. within 20 min.

3. In the presence of 10.0 mM glucose, intracellular $P_i$ levels fell from 40.0 $\mu mol \cdot g^{-1}$ cells dry wt. to 20.0 $\mu mol \cdot g^{-1}$ cells dry wt. in 5 min.

4. Intracellular $P_i$ levels were maintained by 20.0 mM extracellular Tris-$P_i$.

5. $^{45}Ca^{2+}$ uptake was inhibited in $P_i$-depleted cells, even though the metabolic rate (as measured by $Q_{lactate}$) and energy state (as measured by ATP levels) were at acceptable levels [2].

6. Evidence has been presented suggesting that previous reports of glucose inhibition of calcium uptake can be attributed to a competition for available intracellular $P_i$ between glycolytic processes and the mitochondrial calcium uptake mechanism.

## Introduction

Previous studies have shown that glucose and endogenous substrates are equally capable of maintaining ATP levels in intact ELD ascites tumor cells [3]. Thus, the report of Cittadini et al. [1] that, in contrast to energy derived from respiration, energy derived from glycolysis would not support calcium uptake was surprising. Current studies in our laboratories indicate that the energy state as well as inorganic phosphate are important factors in the regulation of calcium

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uptake [4]. In this study, the possible role of $P_i$ in the glucose inhibition of calcium uptake in the ascites tumor cell was examined.

## Materials and Methods

ELD ascites tumor cultures were maintained and harvested as reported earlier [5]. The cells were washed twice with buffered, physiological saline consisting of 10.0 mM (2-*N*-morpholino) propane sulfonic acid (pH 7.4) (United States Biochemical Corporation, Cleveland, Ohio), 100.0 mM NaCl, 6.0 mM KCl, and 2.0 mM $MgCl_2$. To separate the washing medium from the cells, the suspension was centrifuged at 1000 × *g* for 10 min. A 20% cell suspension was prepared in the buffered, physiological saline.

Calcium movements were followed using calcium-45 as a tracer (New England Nuclear Corp., Boston, Mass.). The 20% cell suspension was diluted to 8% in the buffered, physiological saline. Glucose and Tris-$P_i$ (pH 7.4) were added as indicated, the final volume being 5.0 ml. To avoid the possibility of calcium-phosphate artifacts, control experiments were run to determine at what concentrations calcium-phosphate precipitates would occur under the conditions of the experiments. Precipitation was first observed at 1.0 mM $CaCl_2$ and 20 mM Tris-$P_i$ in the presence of buffered, physiological saline. Experiments were carried out at a calcium concentration of 0.5 mM where as high as 30 mM Tris-$P_i$ could be used without visible precipitate. At time zero, $^{45}CaCl_2$ at a specific activity of 1.0 Ci · $mol^{-1}$ was added to a final concentration of 0.5 mM. Cells were incubated at 37°C in a Dubnoff metabolic shaking water bath (Precision Scientific Co., Chicago, Ill.). At the times indicated, 0.5 ml aliquots of the incubation mixture were removed. Each aliquot was washed twice with cold, buffered, physiological saline. The subsequent cell suspension was deproteinized with 6% perchloric acid. After centrifugation for 5 min at 1000 × *g*, the supernatant was examined for $^{45}Ca^{2+}$ content by scintillation counting in Bray's Cocktail with a Beckman LS-230 Scintillation Counter (Fullerton, Calif.).

Free, intracellular $P_i$ levels were monitored using the Lohmann-Jendrassik [6] modification of the Fiske-Subbarow method. The cells for these studies were prepared and incubated as described above. Aliquots of the deproteinized supernatant were examined for $P_i$ content at 660 nm with a Cary 14 M spectrophotometer.

The method of Estabrook and Maitra [7,8] was used in assaying for glucose 6-phosphate and ATP. Samples were prepared as described above; however, aliquots were washed with cold, buffered, physiological saline and kept on ice during the deproteinization process. The deproteinized supernatant was decanted and neutralized with 0.73 M NaOH and 0.15 M triethanolamine buffer (pH 7.5). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1), and nicotinamide-adenine dinucleotide phosphate were obtained from Sigma Chemical Company, St. Louis, Mo.

Lactic acid determinations were based on the Cohen and Noell modification of the Horn and Bruns method [9]. Lactic dehydrogenase (EC 1.1.1.27) was obtained from Sigma Chemical Co., St. Louis, Mo. Nicotinamide-adenine dinucleotide was obtained from Pabst Laboratories, Milwaukee, Wiscon. Samples were read at 366 nm with a Cary 14M spectrophotometer.

Proton production was determined by titrating a 0.5 ml aliquot of the incubation mixture back to the time zero pH with 0.1 M NaOH. A Beckman research pH meter was used for pH readings (Fullerton, Calif.).

## Results

*Glucose supports calcium-45 uptake at 37° C.* In contrast to the findings of Cittadini et al. [1] working anaerobically at 25° C, glucose was shown to support $^{45}Ca^{2+}$ uptake under aerobic conditions at 37° C. Preliminary studies by Charlton in our laboratories demonstrated no appreciable difference between calcium uptake supported by anaerobic glycolysis and calcium uptake supported by aerobic glycolysis. This is not surprising in light of the pronounced Crabtree effect observed in these cells [10]. $^{45}Ca^{2+}$ uptake by ascites tumor cells in the presence of 10.0 mM glucose was measured at different time intervals. A burst of uptake occurred in the first 5 min., followed by a slower accumulation (Fig. 3A). Under optimal conditions, 10.0 mM glucose was able to support the accumulation of up to 5.0 $\mu$mol $^{45}Ca^{2+} \cdot g^{-1}$ cells dry wt. in 20 min.

*Depletion of free, intracellular $P_i$ by glucose.* Glucose was shown to deplete the free, intracellular $P_i$ stores of the ascites cells. In the presence of 10.0 mM glucose, intracellular $P_i$ levels dropped from 40.0 $\mu$mol $\cdot g^{-1}$ cells dry wt. to

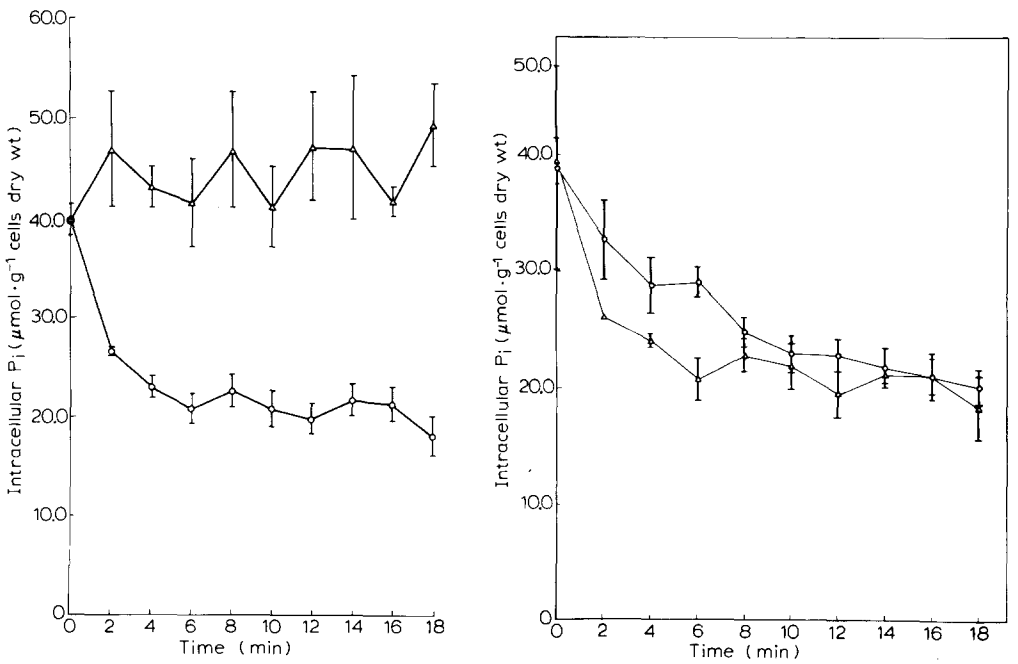


Fig. 1. Glucose depletion and extracellular $P_i$-maintenance of intracellular $P_i$ was measured as a function of time. An 8% cell suspension was incubated at 37° C in the presence of 10.0 mM glucose (○———○) and 10.0 mM glucose plus 30.0 mM $P_i$ (△———△). The soluble fraction of the $HClO_4$ precipitation was assayed for $P_i$ as described in the text. The results presented are the mean values of three experiments.

Fig. 2. Glucose and endogenous substrate depletion of intracellular $P_i$ was measured as a function of time. The 8% cell suspension was incubated at 37° C in the presence (△———△) and absence (○———○) of 10.0 mM glucose. The soluble fraction of the $HClO_4$ precipitation was assayed for $P_i$ as described in the text. The results presented are the mean values of three experiments.

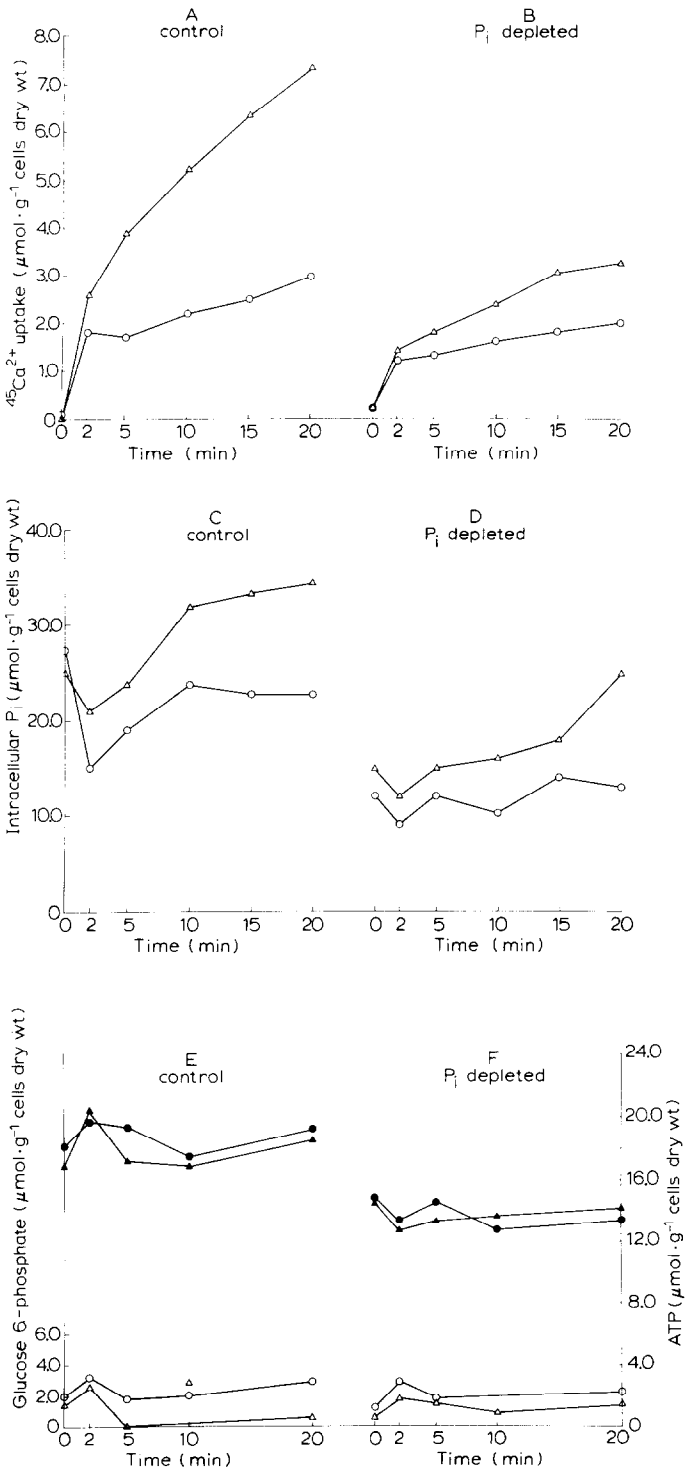


**Fig. 3. See opposite page for legend.**

20.0 $\mu$mol · $g^{-1}$ cells dry wt. within 5 min. The addition of 30.0 mM extracellular Tris-$P_i$ (pH 7.4) maintained free, intracellular $P_i$ at its basal level (Fig. 1). 20.0 mM extracellular Tris-$P_i$ was less effective in this maintenance action during the initial 5 min.

*Depletion of free, intracellular $P_i$ stores by endogenous substrate.* As a control, the effect of endogenous substrates and glucose on intracellular $P_i$ was compared. To our surprise, both were able to deplete intracellular $P_i$ stores. However, in the presence of 10.0 mM glucose, intracellular $P_i$ levels were reduced by half within about 5 min. Endogenous substrates required about 10 min to induce the same reduced $P_i$ level (Fig. 2).

*Artificial depletion of free, intracellular $P_i$.* It was found that if a 20% ascites cell suspension in 154.0 mM NaCl was stored for 45—50 min at 4° C, the free, intracellular $P_i$ levels were reduced by 50%. For 7-day cultures, this represents a drop from 40.0 $\mu$mol · $g^{-1}$ cells dry wt. to 20.0 $\mu$mol · $g^{-1}$ cells dry wt. Examination of the extracellular medium demonstrated that the $P_i$ leaked from the cells.

*$^{45}Ca^{2+}$ uptake supported by glucose with and without added $P_i$ in control and artificially $P_i$-depleted cells.* There has been considerable evidence supporting the idea that the mitochondria act as calcium storage organelles analogous to the sacroplasmic reticulum in muscle [11,12]. In fact, it is quite probable that the mitochondrion acts as the primary regulatory organelle for calcium uptake by intact cells. The importance of $P_i$ in mitochondrial accumulation of calcium has been demonstrated by Lehninger [11]. In light of the above suggestions and findings, it becomes important to examine the role of $P_i$ in the reported glucose inhibition of calcium uptake. $^{45}Ca^{2+}$ uptake in the presence of glucose and glucose plus Tris-$P_i$ in control and $P_i$-depleted cells was examined. In control cells (Fig. 3A) it can be seen that 20.0 mM extracellular Tris-$P_i$ stimulates glucose-supported $^{45}Ca^{2+}$ uptake. The greatest difference in accumulation occurred after the initial 5 min. In artificially $P_i$-depleted cells (Fig. 3B), glucose-supported activity is suppressed in both the presence and absence of $P_i$, although the addition of 20.0 mM Tris-$P_i$ did stimulate to some degree.

To further elucidate what appeared to be $P_i$-dependent glucose inhibition of $^{45}Ca^{2+}$ uptake, intracellular $P_i$ levels, ATP, and glucose 6-phosphate concentrations, lactate production and proton production were assayed using the same cell samples.

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Fig. 3. (A) $^{45}Ca^{2+}$ uptake by fresh ascites tumor cells was measured as a function of time. The 8% cell suspension was preincubated at 37°C for 10 min. At time zero, either 10.0 mM glucose (○———○) or 10.0 mM glucose plus 20.0 mM Tris-$P_i$ (△———△) was added to each flask and the incubation was continued. The results presented are typical of those observed in three experiments. (B) Conditions and procedures were identical to those in (A), except that $P_i$-depleted cells were used. (C) Intracellular $P_i$ levels were monitored as a function of time in the same cell samples as in (A). The effect of 10.0 mM glucose (○———○) or 10.0 mM glucose plus 20.0 mM Tris-$P_i$ (△———△) was determined. (D) Intracellular $P_i$ levels were monitored as a function of time in the same cell samples as in (B). The effect of 10.0 mM glucose (○———○) or 10.0 mM glucose plus 20.0 mM Tris-$P_i$ (△———△) was determined. (E). Glucose 6-phosphate and ATP levels were measured as a function of time in the same cell samples as in (A). The effect of 10.0 mM glucose on glucose 6-phosphate (○———○) and ATP (●———●) as well as the effect of 10.0 mM glucose plus 20.0 mM Tris-$P_i$ on glucose 6-phosphate (△———△) and ATP (▲———▲) was determined. (F) Glucose 6-phosphate and ATP levels were measured as a function of time in the same cell samples as in (B). Conditions were as described in (E).

TABLE I

THE INFLUENCE OF $P_i$ ON LACTATE PRODUCTION

After removing 0.5 ml aliquots of the incubation mixture, the cell suspension was immediately centrifuged at 1000 × *g* for 40 s. The supernatant was decanted and assayed for lactic acid by measuring NADH produced in the conversion of lactate to pyruvate by lactic acid dehydrogenase [9]. The results presented are the mean values, ± standard error of the mean, of three experiments.

| Time (min) | Control | | | $P_i$ depleted | | |
|---|---|---|---|---|---|---|
| | Glucose ($\mu$mol · $g^{-1}$ cells dry wt.) | Glucose + $P_i$ ($\mu$mol.· $g^{-1}$ cells dry wt.) | | Glucose ($\mu$mol · $g^{-1}$ cells dry wt.) | Glucose + $P_i$ ($\mu$mol · $g^{-1}$ (cells dry wt.) | |
| 2 | 13.2 ± 1.0 | 13.8 ± 0.3 | $P < 0.250$ | 6.0 ± 0.1 | 8.3 ± 0.1 | $P < 0.05$ |
| 10 | 64.2 ± 3.3 | 78.8 ± 3.0 | $P < 0.010$ | 46.1 ± 2.0 | 58.3 ± 2.5 | $P < 0.005$ |
| 20 | 126.8 ± 4.6 | 166.9 ± 5.2 | $P < 0.005$ | 88.4 ± 0.4 | 116.9 ± 1.6 | $P < 0.005$ |
| $Q_{lac}$ | 8.5 ± 0.3 | 11.2 ± 0.3 | $P < 0.005$ | 5.9 ± 0.0 | 7.9 ± 0.1 | $P < 0.005$ |

TABLE II

THE INFLUENCE OF $P_i$ ON PROTON PRODUCTION

0.5 ml aliquots of the incubation mixture were removed and immediately measured for pH. Time zero values were used as base points. 20-min samples were titrated back to the time zero pH to determine proton production during this time interval.

| Time (min) | Control | | $P_i$ depleted | |
|---|---|---|---|---|
| | Glucose ($\mu$mol · $g^{-1}$) cells dry wt.) | Glucose + $P_i$ ($\mu$mol · $g^{-1}$) cells dry wt.) | Glucose ($\mu$mol · $g^{-1}$) cells dry wt.) | Glucose + $P_i$ ($\mu$mol · $g^{-1}$ cells dry wt.) |
| 0 | 0 | 0 | 0 | 0 |
| 20 | 257.4 | 382.4 | 211.3 | 278.6 |
| $Q_{H^+}$ ($\mu$l · $mg^{-1}$ cells dry wt. · $h^{-1}$) | 17.3 | 25.7 | 14.2 | 18.7 |
| lac/$H^+$ | 0.47 | 0.43 | 0.42 | 0.42 |

The addition of 20.0 mM Tris-$P_i$ suppresses the initial drop in intracellular $P_i$ levels, and it permits complete recovery of intracellular $P_i$ by 7 min. In the absence of added $P_i$, the data agree with that of Fig. 1, although some recovery is seen (Fig. 3C).

Storage at 4°C in 154.0 mM NaCl resulted in the depletion of intracellular $P_i$ levels by more than 50%. The addition of 20.0 mM extracellular Tris-$P_i$ allows for recovery of intracellular $P_i$ to near control values. In the $P_i$-free system, the low levels of intracellular $P_i$ are maintained. Note the small initial drop in both systems.

Since $^{45}Ca^{2+}$ uptake is dependent on the energy state of the cells [4], it could be argued that the reduced activity could be attributed to low ATP levels in the $P_i$-depleted cells. The ATP concentrations of the cell samples are shown in Figs. 3E and F for the control and $P_i$-depleted systems, respectively. Although the ATP levels fall from about 18.0 $\mu$mol · $g^{-1}$ cells dry wt. to 14.0 $\mu$mol · $g^{-1}$ cells dry wt. when the cells are stored on ice, the levels remain greater than or equal to those reported by Lehninger [13] as being optimal for $^{45}Ca^{2+}$ uptake. *

As a qualitative measure of intracellular $P_i$ which is sequestered by glycolytic intermediates and as an indicator of $P_i$ stimulation of glycolysis, glucose 6-phosphate levels were monitored (Figs. 3E and F). In the control system (Fig. 3E), there is an initial rise in glucose 6-phosphate during the first 2 min indicating a buildup in glycolytic intermediates at control points. The subsequent drop in glucose 6-phosphate demonstrates a stimulation of glycolysis, much more enhanced with added 20.0 mM Tris-$P_i$. A similar pattern is seen in the $P_i$-depleted system; however, the added 20.0 mM Tris-$P_i$ did not appear to stimulate to the same degree as in the control system.

* Calculations were made assuming an average cell diameter of 20 $\mu$m, 3.0 mg cells dry wt. equal to $10^7$ cells, and that at least half of the cellular ATP is available for mitochondrial accumulation of calcium.

A more quantitative determination of glycolytic activity was obtained by measuring changes in lactic acid levels and proton production (Tables I and II, respectively). In each case added extracellular Tris-$P_i$ is seen to stimulate glycolysis. However, there is an overall depression of glycolysis in the $P_i$-depleted cells as would be expected. It is interesting that there is considerably more proton production than lactate release.

## Discussion

Previous investigators have reported that ATP derived from glycolysis was not able to support calcium uptake in ascites tumor cells [1]. The present results indicate that glucose will support calcium uptake and that previous failures to observe calcium accumulation in the presence of glucose may have been due to limited intracellular $P_i$. Evidence is presented suggesting that glucose inhibition of calcium uptake may be due to glycolytic sequestering of free, intracellular $P_i$ stores.

Charlton in our laboratories has clearly demonstrated that calcium uptake in intact cells is blocked by uncouplers such as S-13 (5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide) or rotenone. These data suggest that calcium uptake in intact cells is primarily regulated by mitochondria. Chance and Yoshioka have observed that external $Ca^{2+}$ produces a mitochondrial membrane alkalinization which is neutralized by $P_i$ addition [15]. In intact cells, Charlton has demonstrated a several-fold stimulation of succinate-supported calcium uptake by added $P_i$. The question arises as to how free, intracellular $P_i$ might be involved in mitochondrial calcium uptake. Chappel and Crofts [14] have suggested that a calcium-proton exchange could conceivably be coupled to a phosphate-hydroxyl exchange for a net formation of calcium-phosphate. A phosphate-hydroxyl exchange would prevent an intra-mitochondrial alkalinization. One might also accumulate insoluble calcium phosphate within the mitochondrial matrix when excess phosphate is added. Whatever the mechanism, the question arises as to whether or not a glucose-related process might be competing with the mitochondrial calcium uptake mechanism for available $P_i$.

Initial studies examined the effect of glucose on intracellular $P_i$ levels. Both glucose and endogenous substrate were capable of depleting the intracellular $P_i$ stores, although glucose depletion of $P_i$ was shown to be more rapid (Figs. 1 and 2). Why, then, does one not observe inhibition of uptake with endogenous substrates? Nirenberg [16] has demonstrated the absence of glycogen in ascites tumor cells. Therefore, most endogenous substrate can be presumed to be fatty acids [2]. Since fatty acid catabolism involves mitochondrial $\beta$-oxidation, any $P_i$ depletion is probably due to an increased rate of oxidative phosphorylation, a factor which would enhance calcium uptake. In contrast, glucose-dependent $P_i$ depletion is most likely due to a buildup of phosphorylated intermediates in the cytosol. If a sufficient amount of $P_i$ is sequestered, glucose depletion of $P_i$ would be expected to inhibit calcium uptake.

To test the above hypothesis, a final series of experiments was run examining several parameters in the ascites cells (Figs. 3A—F, Tables I and II). As expected, low intracellular $P_i$ did inhibit calcium uptake. On the basis of glucose-6-phosphate levels, when correlated with free, intracellular $P_i$, it is sug-

gested that the major cause of $P_i$ depletion is a $P_i$-sequestering by glycolytic intermediates. Reversal of glucose-dependent inhibition of calcium uptake by extracellular Tris-$P_i$ could occur in two ways. Racker and co-workers [3] have reported that an increase in the intracellular $P_i$ concentration of 1.0 $\mu$mol · $ml^{-1}$ packed cells will result in a two-fold stimulation of glycolysis *. Thus, a small uptake of extracellular $P_i$, by stimulating glycolysis, could reduce the steady-state level of phosphorylated intermediates [17]. Uptake of extracellular $P_i$ could also be replenishing intracellular $P_i$ stores directly. The former mechanism is most likely responsible for early recovery of intracellular $P_i$, while the latter mechanism would contribute more to the later recovery. The energy state, as reflected by the ATP levels of the cells, does not appear to contribute significantly to glucose inhibition of calcium uptake.

*Q* values based on lactate production or proton production confirm an acceptable metabolic rate in both control and $P_i$-depleted systems [2]. It is worth noting that low intracellular $P_i$ does inhibit glycolysis to some degree, and that extracellular $P_i$ partly overcomes this inhibition. The discrepancy between lactate and proton production can be explained only in part by allowing for proton production during respiration. Further investigation is warranted before a complete explanation is possible.

## Acknowledgment

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* This represents about 20.0 $\mu$mol · $g^{-1}$ cells dry wt.